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Full Length Research Paper

Arbuscular mycorrhizas by contact with mycorrhized *Stylosanthes guianensis* enhance P use efficiency for N₂ fixation in the common bean (*Phaseolus vulgaris* L.)

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Common bean (*Phaseolus vulgaris* L.) genotype Flamingo was inoculated with *Rhizobium tropici* CIAT899 and *Glomus intradices* by mycorrhizal inoculant or by contact with mycorrhized *Stylosanthes guianensis* and grown under sufficient versus deficient phosphorus supply after transfer from initial sand culture, for comparing the effects of double inoculation (by contact or by inoculant) on growth, nodulation, mycorrhization of the roots, phosphorus use efficiency and total nitrogen. Although, the results showed that the double inoculation induced a significant increase in all parameters whatever the phosphorus supply and the cultivation systems in comparison to control and no significant difference between both arbuscular mycorrhizal fungi (AMF) treatments. Significant differences among colonization and nodulation of the roots and growth were found in both hydroaerobic and sand culture. Nevertheless, the highest phosphorus use efficiency and plant total nitrogen were found under P deficiency in both AMF treatments. It is concluded that inoculation with rhizobial and arbuscular mycorrhizal fungi (by contact or by inoculant) could improve symbiotic nitrogen fixation even under phosphorus deficiency.

Key words: Arbuscular mycorrhizal fungi, *Glomus intradices*, nitrogen fixation, *Phaseolus vulgaris*, phosphorus, rhizobia, *Stylosanthes guianensis*, symbiosis.

INTRODUCTION

Phosphorus and nitrogen constitute the most limited nutriment for vegetative growth (Tajini et al., 2009, 2011). In order to assess the capacity of plant to acquire nutrients, arbuscular mycorrhizal fungi and rhizobia are two of the most important plant symbionts. They play a key role in natural ecosystems and influence plant productivity, plant nutrition and improved inhibition of fungal plant pathogens (Demir and Akköprü, 2007; Wehner et al., 2010; Abohater et al., 2011). Mycorrhiza benefits the host through mobilization of phosphorus from non-labile sources, whereas rhizobia fix N₂ (Scheublin

and Vander, 2006). Previous works on the tripartite symbiosis legume-mycorrhiza-rhizobia have shown stimulatory (Edwards et al., 1998; Xiao et al., 2010) or inhibitory (Söderberg et al., 2002; Scheublin and Vander, 2006; Franzini et al., 2010) effects on each other or on the growth of plants.

A few studies have shown that some bacterial species respond to the presence of certain AM fungi (Andrade et al., 1997; Artursson et al., 2006), suggesting a high degree of specificity between bacteria associated with AM fungi. Thus, the specific bacteria together with AM fungi may create a more indirect synergism for plant growth (Barea, 1997) including nutrient acquisition (Barea et al., 2002) and enhancement of root branching (Gamalero et al., 2004). In addition, the AM fungi themselves have also been shown to have an impact on

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the composition of bacterial communities in their mycelium environment (Artursson et al., 2006). The rhizobia-bean symbiosis when in association with arbuscular mycorrhizal fungi (AMF) is known to benefit from a better supply of phosphorus (Sanginga et al., 2000). The AMF is also able to acquire phosphorus in organic form that is not directly assimilated by plants (Bucher et al., 2001). The mechanisms affecting the efficiency of absorption and utilization of phosphorus in plants are related to colonization by mycorrhizae (Jia et al., 2004). Furthermore, Jin et al. (2010) found that dual inoculation with AMF and rhizobia decreased the harmful influence of sulphate salinity on plant growth and nutrient accumulation (P, N and Proline) in *Lathyrus sativus*, compared with the control treatments. Both symbioses share parts of signalling pathways, indicating intimate interactions between all three partners during co-evolution (Demir and Akköpru, 2007; Stancheva et al., 2006; Xiao et al., 2010). On the other hand, Aysan and Demir (2009) reported that the information on the mechanisms controlling interactions of bacteria with AM fungi and plant roots in the mycorrhizosphere and their activities are very difficult to generalize because the interactions involving arbuscular mycorrhiza, root rot fungi and *Rhizobium* vary with the microbial species and plant cultivars.

The present work was undertaken to investigate whether sensitivity of symbiotic nitrogen fixation to phosphorus deficiency was restored by symbiosis with arbuscular mycorrhizal fungi by contact with mycorrhized *S. guianensis* under control conditions.

MATERIALS AND METHODS

The experiment was realized for this work to compare two cases of tripartite symbiosis in hydroaerobic and sand cultures. The common bean genotype was inoculated by mycorrhizal inoculant or by contact with mycorrhized *S. guianensis* or not (control) and in all cases received similar rhizobial inoculation. The experimental design consisted of randomized block with 3 replications. The SAS software (1997) was used to perform the statistical analysis, results were submitted to ANOVA, and comparison of means was achieved by the Duncan's multiple range test ($p \leq 0.05$).

Biological material

The common bean genotype Flamingo was selected on the basis of its tolerance to salinity (Jebara et al., 2001) and came from seeds initially supplied by B. Voyeset from CIAT (Colombia). Seeds were surface-sterilized with 1.3% calcium hypochlorite for 15 min with constant stirring, and then washed with sterile distilled water. They were germinated on 0.8% sterile agar plates for 3 days at 28°C in the dark, and had a germination rate of 80%. Rhizobial inoculation was performed by soaking the seedlings for 45 min in a freshly prepared suspension of *Rhizobium tropici* CIAT899 containing 108 bacteria ml^{-1} .

Seedlings were grown for 2 weeks in 1000 ml pots filled with an autoclaved sand-soil mixture (9: 1 v:v) recolonized with soil bacteria according to Jansa et al. (2002). Treatments used were non-mycorrhizal inoculated or inoculated with AMF. Fifty grams of AMF

inoculums, *Glomus intraradices* BEG157, were placed below the seedlings. The inoculum used for the pots consisted of chopped roots of pot cultures planted with leek (*Allium porrum*) and grown for 18 months in a glasshouse. Pots without AMF inoculums received 50 g of autoclaved AMF inoculums in order to avoid differences in soil nutrient content linked to AMF inoculums additions. Fifty grams of AMF inoculum is approximately equal to 1000 spores of the AMF specie contained at least 20 infective propagules of AMF per gram of chopped root. The amount of mycorrhizae substrate was characterized by low available N (0.007%) and P (0.001%).

In order to assess the mycorrhization by contact, also some surface-sterile seeds were sown and grown for 2 weeks in soil-sand culture in contact with mycorrhized *S. guianensis* by *G. intraradices* BEG157 and thereafter transferred into hydroaerobics vats.

Growth conditions

Trials were performed in a temperature-controlled glasshouse with night/day temperatures of 25/35°C, and a 16 h photoperiod with complementary illumination of 400 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Inoculated seedlings with *R. tropici* CIAT899 and *G. intraradices* inoculant or by contact with mycorrhized *Stylosanthes*. After 2 weeks, one plant only was left in the soil-sand substrate. Pots were watered with distilled water every 2 days until harvest, and once a week received the following nutrient solution: macroelements: K_2SO_4 (1.25 mm), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (2.05 mm), CaCl_2 (3.3 mm); microelements: Fe EDDHA (8.5 $\mu\text{mol Fe as sequestrene}$), H_3BO_3 (4.0 μmol), MnSO_4 (6.0 μmol), ZnSO_4 (0.9 μmol), CuSO_4 (1.0 μmol), NaMoO_4 (0.1 μmol).

After 2 weeks, two plants from sand culture in contact with mycorrhized *Stylosanthes* or for other treatments were transferred into 45 L plastic vats by gently passing them through the hole of a rubber stopper with cotton wool fixed at the hypocotyl level. Each vat containing 20 plants and 20 L of the nutrition solution, and topped up to 45 L with sterile distilled water (Vadez et al., 1996). Plants were grown in the vats and received the aforementioned nutrient solution that was replaced every week: Every week, 75 or 250 $\mu\text{mol KH}_2\text{PO}_4 \text{ pl}^{-1}$ were applied for the deficient or sufficient P treatment, namely 75 P and 250 P. The solution was supplemented with 2 mmol urea plant $^{-1}$ during first two weeks, 1 mmol urea plant $^{-1}$ during the next two weeks and no more urea during the last two weeks. The nutrient solution was constantly aerated at a flow of 400 ml plant $^{-1} \text{ min}^{-1}$. The pH was buffered close to 7 with CaCO_3 (1 g l $^{-1}$).

Assessment of AMF colonization

The plants were harvested after 6 weeks of growth, half of the root system was used for estimation of the extent of root colonization by the AMF as follows : roots were cleared in KOH 10% (w:v) at 80°C for 30 min followed by rinsing with water and two rinses with 1% HCl of 1 h each. Thereafter, the roots were immersed at 80°C for 1.5 h in a staining solution consisting of lactic acid: glycerol: water (1:1:1 v:v:v) and 0.1% of each Trypan Blue and Methylene Blue. After washing away the stain, the roots were de-stained in tap water for 30 min at room temperature. The roots were examined under a compound microscope for quantitative colonization assessment by magnified-intersection (McGonigle et al., 1990).

Biomass and percentages of P and N at harvest

At harvest, shoot, nodules and roots were separated and dried at 70°C for 2 days, and dry weight of each fraction was calculated. The percentage of P was measured in samples of ground tissues

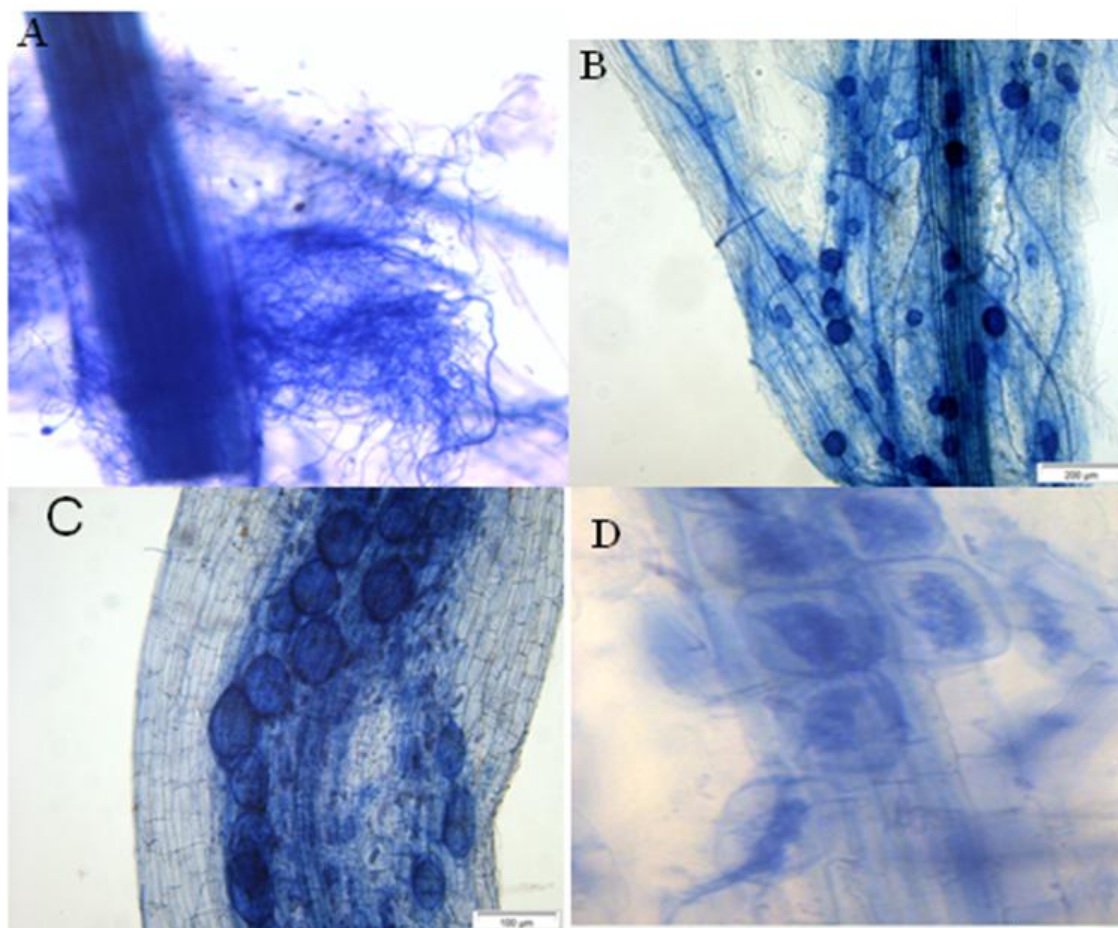


Figure 1. Hyphea (A), Vesicles and Hyphea (B), Vesicles (C) and Arbuscule (D) associated with roots of common bean Flamingo after contact with mycorrhized *Stylosanthes guianensis* by *G. intraradices* in hydroaerobic (A, and B) or in sand-soil culture (C and D).

following wet digestion with nitric-perchloric acid (6: 1, v: v) at 250°C for 6 h, using the phosphovanado-molybdate method (Taussky and Shorr, 1953). Shoot samples of plants inoculated or not with AMF under sufficient (250 $\mu\text{mol P}$ per plant per week) or deficient (75 $\mu\text{mol P}$ per plant per week) and in sand culture. P levels were analyzed. The P use efficiency (PUE) was calculated as the ratio of biomass (shoot + root) g⁻¹ / mean plant P content mg⁻¹. Total nitrogen percentage (TN) was measured by the Kjeldahl procedure on plants harvested for biomass determinations.

RESULTS

Mycorrhization by contact or after inoculation in hydroaerobic- versus sand- cultures

Photographs in Figure 1 show hyphea (A), hyphea and vesicles (B), vesicles (C) and arbuscule (D) associated with roots of common bean Flamingo after contact with mycorrhized *Stylosanthes* by *G. intraradices* in hydroaerobic (A and B) or in sand culture (C and D). Data in Figure 2 show that the root-colonization was decreased by P supply since the colonization rates by all

structures colonization were higher under P deficiency than under P sufficiency in both treatments, though; there was only significant difference in hyphae and vesicles colonization after contact with mycorrhized *Stylosanthes*. Nevertheless higher colonization-rates of all structures colonization were observed in sand culture, either after contact with mycorrhized *Stylosanthes* or after inoculation by arbuscular mycorrhizal fungi (AMF) but with no marked significant difference between both AMF treatments.

Nodulation

Data in Figure 3 show that the nodulation was affected by cultivation system ($p \leq 0.001$) and by AMF treatments ($p \leq 0.01$). Thus, in both AMF treatments, the interaction tripartite symbiosis induced an increase in nodule number and nodules biomass as compared to non-AMF-inoculated plants whatever the cultivation system or the P supply. The highest nodule number per plant was observed under P sufficiency with both AMF treatments

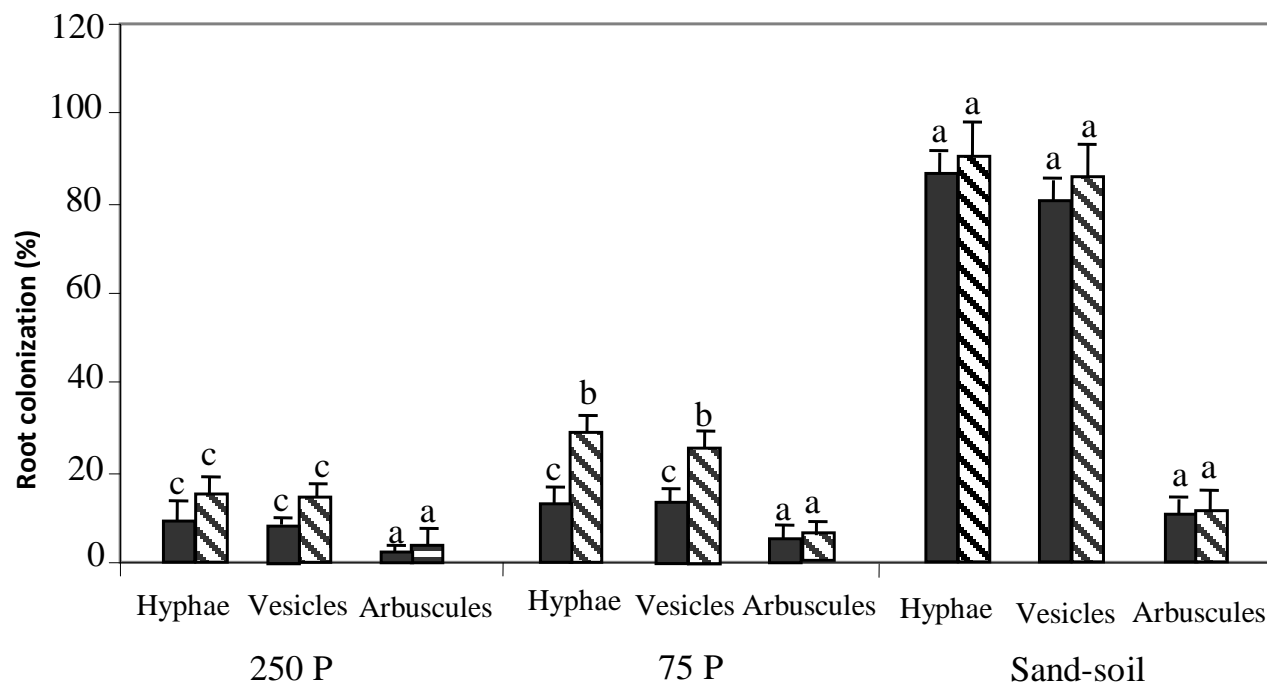


Figure 2. Effect of *G. intraradices* on root colonization by hyphae, vesicles and arbuscules of common bean genotype Flamingo, grown in sand-soil and in hydroaeroponic culture under P sufficiency (250 P) versus P deficiency (75 P) after inoculation with *R. tropici* CIAT899, and with *G. intraradices* by mycorrhizal inoculant (black bars) or by contact with mycorrhized *Stylosanthes guianensis* (hatched bars) both in sand pre-culture. Data are means \pm SD of 3 replicates, plants harvested at 50 days after sowing. For each structure, different letters indicate significant differences between treatment means according to Duncan's multiple range test ($p \leq 0.05$). P= phosphorous.

(Figure 3A). AMF treatments induced an increase in nodule number of 54 and 70% in sand culture, 68 and 73% under P sufficiency and of 70 and 68% under P deficiency for plants grown in contact with mycorrhized *Stylosanthes* and inoculated plants by AMF, respectively, in comparison with control plants (Figure 3A). Also, AMF increased significantly the nodule mass per plant of 15 and 43% in sand culture and 25 and 45% under P sufficiency in plants grown in contact with mycorrhized *Stylosanthes* and inoculated plants, respectively, in comparison with control plants (Figure 3B). By contrast, under P deficiency there was no significant difference in biomass of nodules whatever the AMF treatment or non-AMF-inoculated plants (Figure 3B).

Shoot and root growth

Data in Figure 4 show that plant-growth was slightly increased by AMF inoculation since higher dry weight was observed for plants inoculated with AMF than for control plants ($p \leq 0.003$) whatever the AMF treatments and cultivation system. Systematically higher dry weight of plants was observed in hydroaeroponics culture than in sand culture. In the other hand, there was no significant difference in shoot growth between AMF by contact with

mycorrhized *Stylosanthes* and by AMF inoculation treatments whatever the cultivation system (Figure 4A). Thus, AMF induced a significant increase in shoot dry weight of 14 and 24% in sand culture, 22 and 25% under P sufficiency and 10 and 15% under P deficiency for plants grown in contact with mycorrhized *Stylosanthes* and inoculated plants by AMF, respectively, in comparison to that in control plants.

Root biomass was also affected by inoculation with AMF ($p \leq 0.04$) (Figure 4B). In comparison to control plants, AMF induced a significant increase of 39 and 48% in root-growth in sand culture and 26 and 21% under P sufficiency in plants inoculated by contact with mycorrhized *Stylosanthes* and by AMF inoculation, respectively, in comparison to that in control plants. But, with deficient P no significant effect in root-growth in plants inoculated by contact with mycorrhized *Stylosanthes* and control plants (Figure 4B). For control plants, the lowest root-growth was observed in sand culture.

N and P accumulation and P use efficiency

Data in Figure 5A show that shoot nitrogen percentage varied with the cultivation systems ($p \leq 0.005$), the P

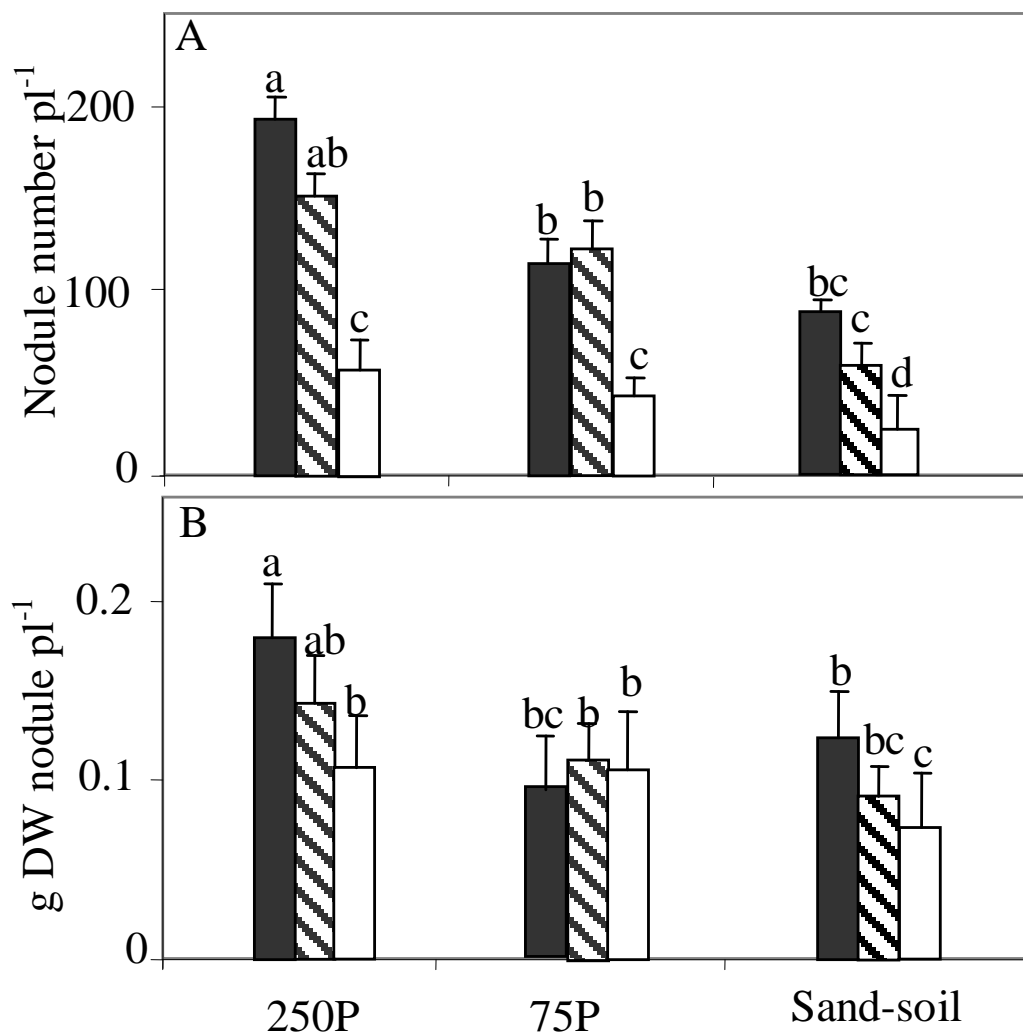


Figure 3. Effect of *G. intraradices* on number (A) and dry weight (B) of nodules of common bean genotype Flamingo, grown in sand-soil and in hydroaeronic culture under P sufficiency (250 P) versus P deficiency (75 P) after inoculation with *R. tropici* CIAT899, and with *G. intraradices* by mycorrhizal inoculant (black bars) or by contact with mycorrhized *Stylosanthes guianensis* (hatched bars) or not as control (open bars). Data are means \pm SD of 3 replicates, plants harvested at 50 days after sowing; different letters indicate significant differences between treatment means according to Duncan's multiple range test ($p \leq 0.05$).

supply ($p \leq 0.001$) and was altered by AMF treatments ($p \leq 0.001$). Thus, the AMF in combination with CIAT899 was induced a significant increase in shoot nitrogen percentage in comparison to control plants, whereas there was no significant difference between both AMF treatments for each P treatment and for each cultivation system (Figure 5A). With AMF, the percentage of nitrogen increased significantly of 29% in sand culture with both AMF treatments. Under P sufficiency, the increases of about 57 and 51% were observed in plants inoculated by contact with mycorrhized *Stylosanthes* and by AMF inoculation, respectively, in comparison to that in control plants. But, under P deficiency, the significant increase was 69 and 66% for plants inoculated by contact

with mycorrhized *Stylosanthes* and by AMF inoculation, respectively, in comparison with control plants (Figure 5A).

Regarding phosphorus percentage, significant differences between cultivation system ($p \leq 0.001$) and AMF treatments ($p \leq 0.006$) were recorded (Figure 5B). The highest phosphorus percentage was observed in plants grown with mycorrhized *Stylosanthes* under P sufficiency with a mean of 0.53% which not significantly differed from 0.47% in inoculated plants. By contrast, the lowest phosphorus percentage was obtained in sand culture with means of 0.18% with both AMF treatments.

Thus, AMF induced a significant increase of 56 and 52% under P sufficiency which significantly differed from

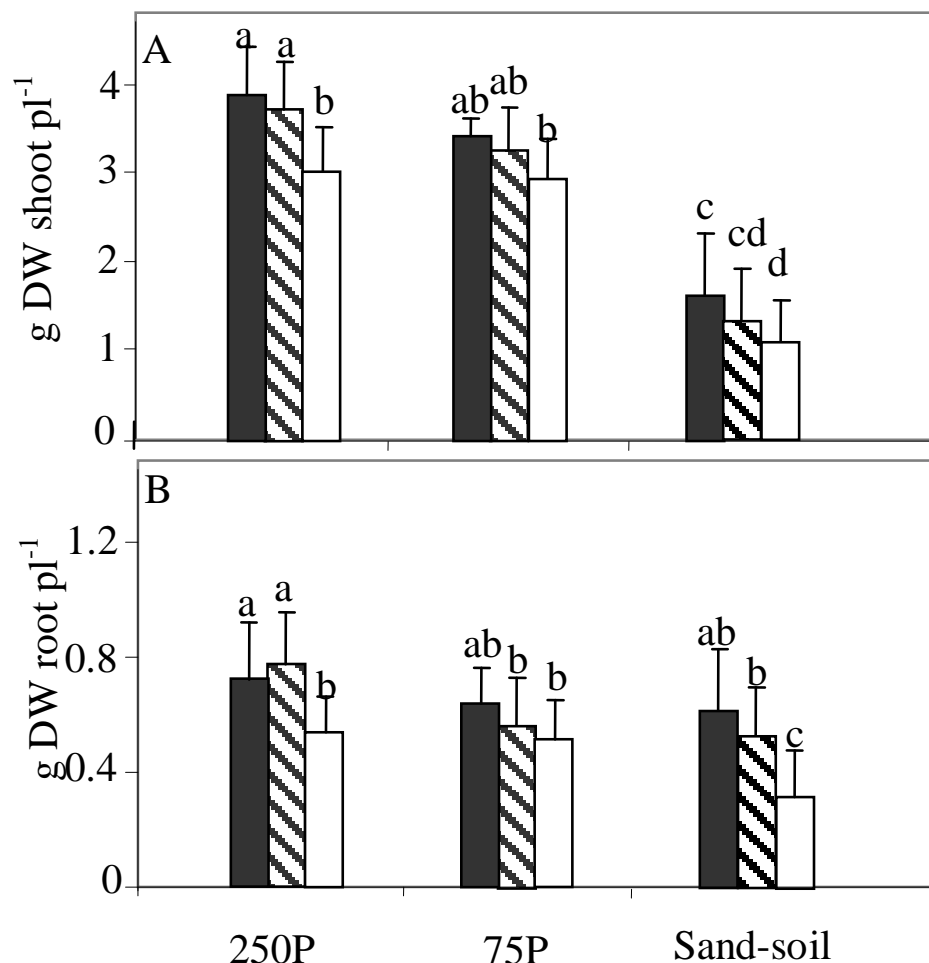


Figure 4. Effect of *G. intraradices* on dry weight of shoot (A) and root (B) of common bean genotype Flamingo, grown in sand-soil and in hydroaeronic culture under P sufficiency (250 P) versus P deficiency (75 P) after inoculation with *R. tropici* CIAT899, and with *G. intraradices* by mycorrhizal inoculant (black bars) or by contact with mycorrhized *Stylosanthes guianensis* (hatched bars) or not as control (open bars). Data are means \pm SD of 3 replicates, plants harvested at 50 days after sowing; different letters indicate significant differences between treatment means according to Duncan's multiple range test ($p \leq 0.05$).

that under P deficiency (7 and 14%) in plants grown with mycorrhized *Stylosanthes* and in inoculated plants, respectively (Figure 5B). It was clear that P use efficiency was strongly affected by P supply ($p \leq 0.04$) and AMF ($p \leq 0.001$) treatments in the common bean. Figure 5C shows the increase of P use efficiency in comparison to control plants and no significant difference between both AMF treatments. Indeed, AMF significantly increased the P utilization efficiency to 58% in sand culture. Also in hydroaeronomics, plants grown with mycorrhized *Stylosanthes* showed increases of about to 38 and 33% under P sufficiency versus P deficiency, respectively. By contrast, in plants inoculated with *Glomus* the increases of about to 34% and 25% in 250P versus 75P, respectively. The highest P use efficiency was observed in plants grown with mycorrhized *Stylosanthes* under P

deficiency with a mean of 0.14 ± 0.03 g DW mg^{-1} P. The lowest P utilization was obtained under P sufficiency in plants inoculated or not with AMF (Figure 5C).

DISCUSSION

The interesting finding of this study is the significant growth benefits of the tripartite symbiosis common bean-rhizobia- arbuscular mycorrhizal fungi (AMF) in both cultivation system whatever the AMF treatments (by contact with mycorrhized *Stylosanthes* or by AMF inoculant). The observation showed that the inoculation of common bean with CIAT899 did not restrict AMF entry or its roots colonization of bean plants, these data agrees with the previous observations of Tajini et al. (2009).

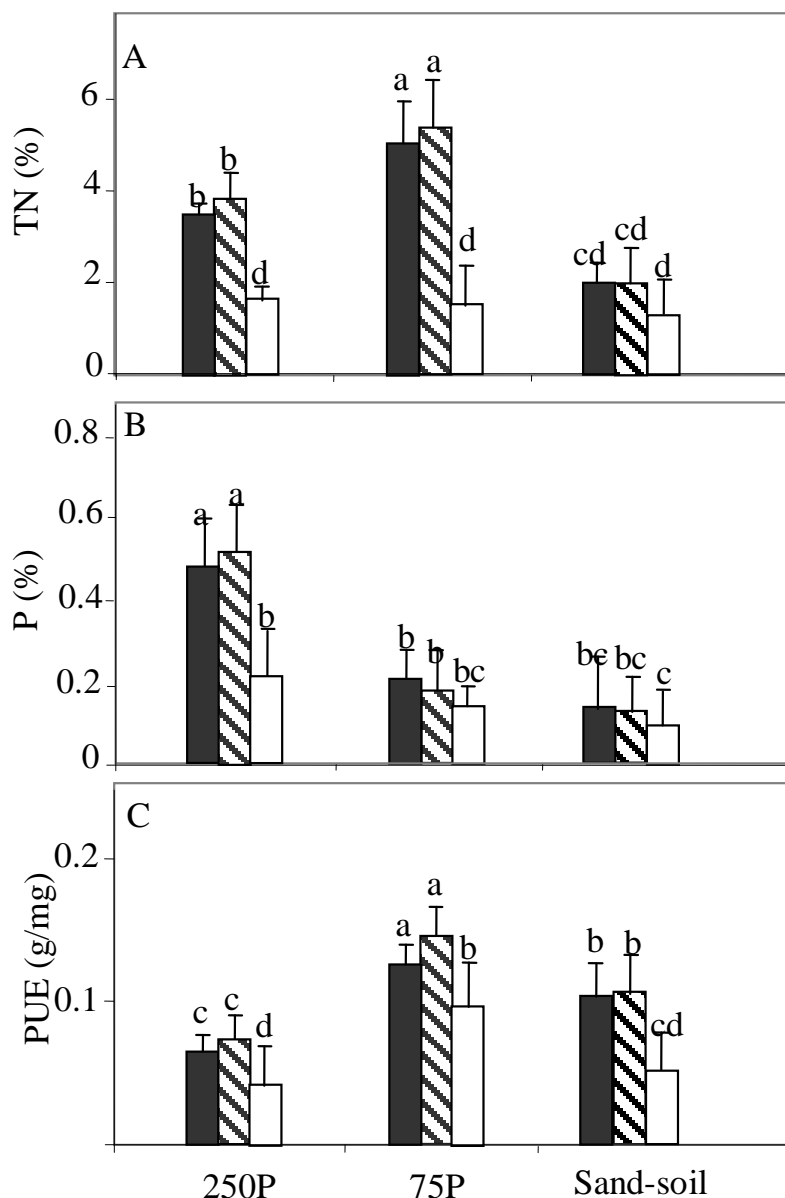


Figure 5. Effect of *G. intraradices* on shoot nitrogen percentage (A), shoot phosphorus percentage (B) and phosphorus use efficiency (C) of common bean genotype Flamingo, grown in sand-soil and in hydroaeronic culture under P sufficiency (250 P) versus P deficiency (75 P) after inoculation with *R. tropici* CIAT899, and with *G. intraradices* by mycorrhizal inoculant (black bars) or by contact with mycorrhized *Stylosanthes guianensis* (hatched bars) or not as control (open bars). Data are means \pm SD of 3 replicates, plants harvested at 50 days after sowing; different letters indicate significant differences between treatment means according to Duncan's multiple range test ($p \leq 0.05$).

In both AMF treatments, the higher colonization rate of roots was found in sand-soil culture, where P limited conditions are more pronounced, than in hydroaeronic systems (Figure 2). It is believed that mycorrhizae especially had positive effects on plants grown in soils where P is likely to limit plant growth by

increasing the soil volume explored by AM hyphae relative to that of root hairs of non-AM plants. This would agree with previous studies showing highest mycorrhizal benefits to plant growth under moderate conditions and P deficiency (Mathimaran et al., 2005; Tajini et al., 2009; Faghire et al., 2010; Xiurong et al., 2011), especially, with

leguminous plants harbouring a coarser root system with less extension of root hairs than graminaceous (Isobe and Tsuboki, 1998). This difference in colonization rates between sand and hydroaeroponic culture could be explained by the orthophosphate (Pi) inhibiting AMF colonization (Grant et al., 2005). This could either be due to direct limitation by Pi in the solution, or to indirect limitation due to the better P status of the plants.

In the hydroaeroponic culture where P levels are replenished weekly, shoot growth and N₂ fixation appear to be controlled by the efficiency in P utilization as previously observed by Rodino et al. (2009) and Bargaz et al. (2011a, b). The highest phosphorus use efficiency and nitrogen accumulation in the present work was obtained in the plants grown with mycorrhized *Stylosanthes* or in plants inoculated by *Glomus* (Figures 5A and C) illustrates the relationship between P use efficiency and symbiotic nitrogen fixation (Tang et al., 2001; Tajini et al., 2011) and demonstrates that the mechanisms affecting the efficiency of absorption and utilization of phosphorus in plants are related to colonization by AMF (Bucher et al., 2001; Jia et al., 2004). It was also indicated that the shoot growth and N₂ fixation were determined mainly by the efficiency in P utilization (Rodino et al., 2009; Bargaz et al., 2011a, b).

Previous studies with common bean found that nitrogen fixation was significantly limited by P deficiency (Vadez and Drevon, 2001; Bargaz et al., 2011a, b). This was not the case of our study (Figure 5A) and this could be attributed to the effect of inoculation with both rhizobia and AMF whatever the cultivation systems.

Plants deficient in P show decreased nodule-number (Vadez and Drevon, 2001), and biomass when grown in soil, sand and alkaline solution, or hydroaeroponics (Araújo et al., 1997; Bargaz et al., 2011a, b; Mandri et al., 2011; Vadez and Drevon, 2001). The present study provides additional evidence that nodulation could be improved by dual inoculation in plants grown with mycorrhized *Stylosanthes* or in plants inoculated by *Glomus* under P deficient conditions. In addition, AM fungi improve plant growth parameters and nutrient uptake, but no significantly effect was pronounced between both AMF treatments.

The obtained results indicated that both AMF treatments significantly increased nitrogen accumulation in the shoot and increased the phosphorus content and phosphorus use efficiency in plants, compared with their controls which were not dually inoculated (Figure 5). These findings are in agreement with that of Aysan and Demir (2009), Askar and Rashad (2010) and Xiurong et al. (2011). It is well known that AM fungi can improve the nutrient status of their host plants (Smith and Read, 2008; Tajini et al., 2009; Kim et al., 2010). It is also thought that the plant-rhizobium system benefits from the presence of AM fungi because the mycorrhizae ameliorate not only P deficiency but also any other nutrient deficiencies that might be limiting to rhizobium (Smith, 2002). Similarly, Nautiyal et al. (2010) found that

dual inoculation of *Cicer arietinum* L. with rhizobia and AMF significantly enhanced the number of nodules and the dry weight per plant. Kim et al. (2010) showed that the combined inoculation of *Methylobacterium oryzae* strains and AM fungi in soil culture increased plant growth, resulted in significantly higher nitrogen accumulation in roots as well as shoots, and increased the phosphorus content of red pepper plants compared with uninoculated controls. However, under drought stress inoculation with AM fungi and rhizobial strains inhibited of nodule development and N₂ fixation, and caused a decrease in plant growth (Franzini et al., 2010). Lisette et al. (2003) reported that co-inoculation with rhizobia and compatible AM fungi could dramatically enhance pea growth, and N and P uptake. Therefore, the AM fungi we used for the present study are compatible with the rhizobial strain and common bean genotype, which might have potential for agricultural application.

In conclusion, suitable combinations of AM fungi (by contact or by inoculation) and rhizobia may increase the plant growth and the P use efficiency, enhancing N₂ fixation under limited P supply conditions. For the successful application of these results in biotechnology, multi location field trials are needed to determine the most efficient tri-partite symbioses. These results show an interesting practical application for agricultural development in marginal lands that are often deficient in P. The present study opens possibilities for in-situ non-destructive studies of (i) energy balance in terms of carbon and oxygen requirements for symbiotic respiration, (ii) metabolic monitoring (NMR), and (iii) molecular analyses with in-situ hybridization and RT-PCR with particularly clean material.

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